Golf Course Maintenance and Amphibian Conservation

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Goals: Laboratory Studies- *1) To test the relative toxicity of the most commonly used pesticides (insecticides, fungicides and herbicides) with three diverse taxa of amphibians. 2) To develop a more complete and biologically realistic testing protocol including: a) multiple species; b) short term acute and long term chronic tests; c) multiple life history stages; d) multiple indicators of biological impact; and e) an environment that provides the opportunity to detoxify or potentate chemicals in a more biologically realistic way.*

Field Studies- *1) To access the feasibility of "stocking" wetlands in order to establish breeding populations of desired amphibian species. 2) To evaluate the relative success of small temporary wetlands versus larger permanent bodies of water more typical of golf course design.*

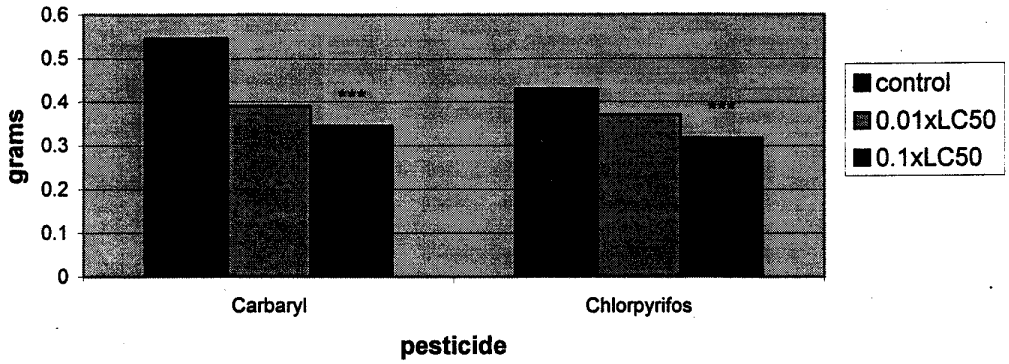
Laboratory study

The toxicity of three pesticides (carbaryl, chlorpyrifos, and imidacloprid) was investigated using American toad tadpoles (*Bufo americanus*). These trials were completed by August and the data analyzed by October. Effects on survival, growth, and time to metamorphosis analysis were consistent with previous results obtained using chorus frogs (*Pseudacris triseriata*). Concentrations of pesticide had a significant effect on survival. Prior to initiation of the *Bufo* trial, the LC50 (concentration of pesticide needed to kill 50% of test organisms) was determined for each pesticide. The estimated LC50s in parts per billion were 468,000 for imidacloprid, 63,167 for carbaryl, and 1316 for chlorpyrifos. All tadpoles placed in high (0.5xLC50) concentrations died during the trial whereas survival of tadpoles at all other concentrations was above 95%. Subsequent analyses were performed only on medium (0.1xLC50), low (0.01xLC50) concentrations and controls. Growth of tadpoles was significantly ($P<0.05$) decreased by chronic exposure to 0.1xLC50 concentrations of carbaryl and chlorpyrifos (Figure 1). Significant differences between control tadpoles and those raised in medium concentrations were also observed in time to metamorphosis (measured as day front limbs emerge). Tadpoles in medium concentrations of all pesticides took an average of three days longer to reach metamorphosis when compared with controls. Sublethal effects on time to metamorphosis and growth would be expected to have negative effects on population persistence.

Water and sediment samples collected during the *Pseudacris* larval trial were analyzed for chlorpyrifos by The Institute of Wildlife and Environmental Toxicology. Results indicate that pesticide is rapidly absorbed by the sediment. For example, chlorpyrifos was added to a tank at a concentration of 112 ppb, and 24 hours later the concentration in water was found to be 23 ppb. At termination of the experiment (after 4 repeated applications of pesticide), the concentration of chlorpyrifos in the sediment was 443 ppb. Because many frog tadpoles feed in the substrate and are detritivores, this pathway may be a more important contributor than pesticide residues in the water column.

Summary: Larval trials on *Bufo* were completed and data indicates that pesticide concentrations have effects on survival, growth, and time to metamorphosis of tadpoles which are significant and similar to previous trials on the genus *Pseudacris*. Sediment analysis indicates that pesticide added to the water column becomes concentrated in the sediment. Larval trials using *Rana* are in progress.

Figure 1. The effects of carbaryl and chlorpyrifos on average growth per tadpole (g) after three weeks. Treatments marked * are significantly different from controls.**



Field Studies

In March of 1998, egg masses of *Ambystoma jeffersonianum* (Jefferson salamander) and *Pseudacris triseriata* (chorus frog) were translocated into each of the six experimental ponds. Estimates indicate that 90.7% of *A. jeffersonianum* and 64.5% of *P. triseriata* larvae had successfully hatched. After larvae metamorphosed, individuals were captured by pitfall traps, funnel traps, or time constrained searches. Captured individuals were marked via toe clipping and/or freeze branding for identification in subsequent seasons. Experimental ponds, as well as ponds located on the Rocky Gap Golf Course, have been monitored for natural colonization by local amphibian species. Egg masses found have been identified to species, total number of eggs has been estimated, and location of deposition within experimental ponds has been mapped. Larvae that have been dipnetted have also been identified to species. In the spring and summer of 1998 the following species (in addition to the introduced species) had colonized experimental ponds: *Rana clamitans* (green frog), *Rana sylvatica* (wood frog), *Bufo americanus* (American toad), *Pseudacris crucifer* (spring peeper), *Hyla veriscolor* (gray treefrog), and *Notophthalmus viridescens* (red-spotted newt). Egg masses and/or larvae of the following species have been found on ponds associated with the golf course at Rocky Gap: *Rana catesbeiana* (bullfrog), *R. clamitans*, *B. americanus,* and *P. crucifer*. Although the species composition of experimental and golf course ponds seem similar, several important distinctions should be clarified. The absence of *R. catesbeiana* colonization in experimental ponds should aid in the colonization of smaller species of frogs because *R. catesbeiana* has been implicated in the local extirpation of smaller species due to predation. In addition, ponds associated with the golf course seem not to be colonized as ubiquitously by smaller frog species as experimental ponds are. For instance, not only have we failed to find evidence of *H. versicolor* breeding in golf course ponds, but larvae of *P. crucifer* have only been found in only one golf course pond while all experimental ponds have contained them. The design of our experimental ponds may promote colonization success of some species. Nearly 60% of egg masses were deposited on narrow pond shelves designed to support vegetation that, in part, provides structure for the ovoposition of amphibian egg masses. Similarly, the one golf course pond constructed with a shallow shelf on its perimeter is the only course pond that has shown evidence of *B. americanus* and *P. crucifer* colonization.

Summary: Egg masses of two species of amphibians have been translocated into experimental ponds at Rocky Gap State Park. Hatching success has been monitored in the egg masses and metamorphosed individuals of both species have been captured and marked for future identification. Experimental ponds, as well as golf course ponds, have been monitored for natural colonization of amphibian species. We have detected six amphibian species that use experimental ponds for breeding and we

have detected four species that use golf course ponds for breeding. More importantly, the species composition of our ponds suggests that golf course ponds lack the colonization of smaller species of frogs, while they support the colonization of a large species (i.e. *R. catesbeiana*) that prey on (and could extirpate) smaller species.

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Laboratory Study

We are continuing our investigation of the effects of three insecticides (Carbaryl, Chlorpyrifos, and Imidicloprid) on three genera of amphibians: *Rana, Pseudacris,* and *Bufo.*

To investigate multiple life history stages and relative toxicities, experiments on the impacts of pesticides were divided into two separate trials: egg hatching and larval survival and growth. Before initiation of these trials, LC50s (the concentration of pesticide needed to kill 50% of the test organisms) were determined for each pesticide. The calculated LC50s were used to determine the concentrations for the following experiments. In both the egg-hatching and larval trials four dosages (control, 0.5 x LC50, 0.1 x LC50, and 0.01 x LC50) were evaluated. Relative toxicities of each chemical were tested on each genus. Differences in life stage sensitivity were indicated by testing with chronic exposures. In the egg hatching trials, egg masses were placed in 5 gallon aquaria containing pesticide and maintained until several days post-hatching. In the larval trials, larvae were maintained in 20 gallon aquaria from two weeks post-hatching through metamorphosis.

Multiple indicators of biological impact were evaluated in both egg hatching and larval trials. In the egg hatching trials, hatching success and the number of hatchlings with deformities were recorded. Survival, growth, time to metamorphosis, activity levels, and behavioral and developmental abnormalities of tadpoles were monitored during the larval trials.

To make our experiment more biologically realistic, uncontaminated sediment was placed in the bottom of each aquarium to absorb pesticides and provide an additional pathway for its uptake in tadpoles. In addition, the larval experiment was designed as a static test with pesticide renewal every two weeks in conjunction with water replacement. This simulated repeated pesticide applications and subsequent runoff into wetlands.

LC50's have been performed on four different species and the results (Table 1) indicate that species differ in sensitivity to each pesticide.

Table 1. LC50's (parts per billion) for Carbaryl, Chlorpyrifos, and Imidacloprid.

	Pesticide		
Species	Imidicloprid	Carbaryl	Chlorpyrifos
Bufo americanus	468,000	63,167	1316
Rana catesbeiana	175,500	45,294	273
Rana berlandieri	184,500	51,581	1125
Pseudacris triseriata	388,500	58,075	1125

Hatching success of all genera with all treatments is depicted in Table 2. Analysis of variance indicated that the only pesticide treatment showing significant ($P<0.05$) differences in hatching success was carbaryl at high (0.5xLC50) concentrations. Average percent *Bufo* eggs (54.86) and *Ambystoma* eggs (1.65) hatched in high concentrations of carbaryl was significantly lower than percent eggs hatched in all other treatment combinations. Percent hatchlings exhibiting gross deformities is shown in Table 3. Analysis of variance revealed that *Pseudacris* tadpoles hatched in 0.5xLC50 concentrations of all pesticides show a significantly higher percent deformities (21.22) than tadpoles hatched in $0.1xLC_{50}$ concentrations (7.73).

Table 2. Average percent eggs from each genera hatched in treatments.

		Genera			
Pesticide	Concentration	*Rana*	*Pseudacris*	*Ambystoma*	*Bufo*
Carbaryl	Control	47.35	86.15	67.34	92.41
	0.01xLC50	44.99	89.41	59.69	93.15
	0.1xLC50	50.87	84.66	45.69	96.24
	0.5xLC50	22.14	60.02	1.65*	54.86*
Chlorpyrifos	Control	48.48	78.46	58.49	96.93
	0.01xLC50	51.44	77.96	56.82	98.91
	0.1xLC50	45.69	83.3	55.01	97.42
	0.5xLC50	56.92	80.94	60.24	95.87
Imidicloprid	Control	51.01	82.72	61.57	95.8
	0.01xLC50	48.05	79.39	53.79	98.58
	0.1xLC50	39.27	75.23	51.49	95.71
	0.5xLC50	52.09	82.66	59.71	93.82

Larval trials using the genus *Pseudacris* were completed in April and analysis performed in May. Survival of tadpoles to completion of the experiment is shown in Table 4. As expected, analysis of variance showed significantly lower survivorship of tadpoles in 0.5xLC50 concentrations of all pesticides. Because the sample size of surviving tadpoles in high concentrations was so small, statistical analysis on all other factors was performed only using control, 0.01xLC50 (low), and 0.1xLC50 (medium) concentrations of pesticides.

Table 3. Average percent hatchlings of all genera exhibiting deformities.

		Genera			
Pesticide	Concentration	*Rana*	*Pseudacris*	*Ambystoma*	*Bufo*
Carbaryl	Control	2.7	8.2	6.68	3.07
	0.01xLC50	6.07	13.47	2.4	1.38
	0.1xLC50	1.89	8.87	1.33	4.15
	0.5xLC50	5.59	19.62	-	13.38
Chlorpyrifos	Control	1.47	9.76	3.04	2.77
	0.01xLC50	0.83	14.77	3.62	3
	0.1xLC50	2.41	10.16	4.26	4.96
	0.5xLC50	2.89	19.6	4.64	4.88
Imidacloprid	Control	4.39	9.19	3.07	3.12
	0.01xLC50	1.24	1.25	2.74	2.93
	0.1xLC50	0.8	4.17	4.29	0.73
	0.5xLC50	1.6	24.44	5.5	2.34

Table 4a. Total number of *Pseudacris* tadpoles surviving to metamorphosis.

Pesticide	Concentration			
	Control	0.01xLC50	0.1xLC50	0.5xLC50
Carbaryl	39	40	39	0
Chlorpyrifos	38	40	36	6
Imidicloprid	40	40	39	0

Table 4b. Total number of *Bufo* tadpoles surviving to metamorphosis.

Pesticide	Concentration			
	Control	0.01xLC50	0.1xLC50	0.5xLC50
Carbaryl	39	40	38	0
Chlorpyrifos	40	40	39	0
Imidicloprid	40	40	38	0

Average growth per tadpole of *Pseudacris* in all treatments is given in Table 5. Analysis of variance revealed no significant differences between treatments.

Table 5. Average growth (grams) per tadpole of *Pseudacris*.

	Concentration		
Pesticide	Control	0.01xLC50	0.1xLC50
Carbaryl	0.466	0.379	0.379
Chlorpyrifos	0.502	0.448	0.387
Imidicloprid	0.419	0.497	0.41

Average days to metamorphosis of tadpoles in each treatment type is affected similarly by all pesticides. Average days to metamorphosis of *Pseudacris* tadpoles raised in medium concentrations of all pesticides (38.46) is significantly higher than average days to metamorphosis of tadpoles at low (36.94) and control (36.11) concentrations.

Developmental abnormalities were observed in only two tanks, both containing chlorpyrifos at a concentration of 0.5xLC50 concentration. In these tanks, tadpoles were observed with stunted and malformed limbs.

Behavioral effects of pesticides were observed by calculating activity levels and testing avoidance response of tadpoles in each tank. Abnormal behavior was also noted. Overall activity levels of *Pseudacris* are shown in Table 6. Incidences of abnormal behavior were infrequent (5 total) and no statistically significant differences in avoidance response among treatments was observed.

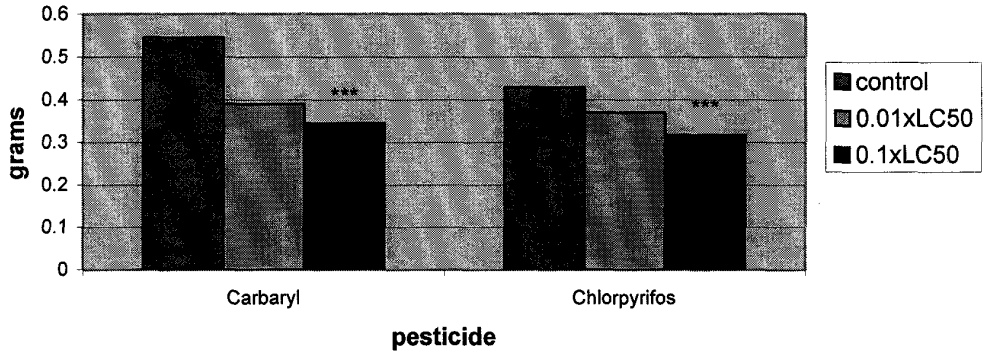
Table 6a. Average percent *Pseudacris* tadpoles active during observation period.

	Concentration		
Pesticide	Control	0.01xLC50	0.1xLC50
Carbaryl	11.49	12.72	10.58
Chloropyrifos	20.28	11.69	16.04
Imidicloprid	14.03	15	14.14

Larval trials using American toad tadpoles (*Bufo americanus*) were completed by August. Effects on survival, growth, and time to metamorphosis were consistent with previous results obtained using chorus frogs (*Pseudacris triseriata*). Concentrations of all pesticides had a significant effect on survival. All tadpoles placed in high (0.5xLC50) concentrations died during the trial whereas survival of tadpoles at all other concentrations was above 95% (Table 4). Subsequent analyses were performed only on medium (0.1xLC50), low (0.01xLC50) concentrations, and controls.

Growth of *Bufo* tadpoles was significantly ($P<0.05$) decreased by chronic exposure to 0.1xLC50 concentrations of carbaryl and chlorpyrifos (Figure 1). No growth differences were observed between control and 0.1xLC50 concentrations of imidacloprid.

Figure 1. The effects of carbaryl and chlorpyrifos on average growth per tadpole (g) after three weeks. Treatments marked * are significantly different from controls.**



Significant ($P<0.05$) differences between control tadpoles and those raised in medium concentrations were also observed in time to metamorphosis (measured as day front limbs emerge). Tadpoles in medium (0.1xLC50) concentrations of all pesticides took an average of three days longer to reach metamorphosis when compared with controls. These results are very similar to those obtained from the *Pseudacris* trial (Table 7). Sublethal effects on time to metamorphosis and growth would be expected to have negative effects on population persistence.

Table 7a. Average days to metamorphosis for *Pseudacris triseriata*.

Pesticide	Concentration		
	Control	0.01xLC50	0.1xLC50
Carbaryl	36.11	36.95	38.41
Chlorpyrifos	35.62	38.55	39.74
Imidicloprid	36.61	35.33	37.22
Average	36.11	36.94	38.46

Table 7b. Average days to metamorphosis for *Bufo americanus*.

Pesticide	Concentration		
	Control	0.01xLC50	0.1xLC50
Carbaryl	36.15	37.25	39.89
Chlorpyrifos	35.08	36.67	38.65
Imidicloprid	36.22	35.05	37.85
Average	35.82	36.32	38.77

Analysis of behavior is continuing but few incidences of abnormal behavior were recorded during the *Bufo* trial and no statistically significant differences among treatment groups is expected for overall activity levels or avoidance response. Unlike *Pseudacris*, no developmental abnormalities were observed among *Bufo* larvae at any time.

Water and sediment samples collected during both larval trials were analyzed by The Institute of Wildlife and Environmental Toxicology. Results indicate that carbaryl and clorpyrifos are rapidly absorbed by the sediment (analysis of imidacloprid is not yet complete). Analyses of water samples taken from tanks containing carbaryl indicate that pesticide disappears from the water column in tanks with sediment within 72 hours (Table 8). Pesticide also appears to bioconcentrate in the sediment. For example, chlorpyrifos was added to a tank at a concentration of 112 ppb, and 24 hours later the concentration in water was found to be 23 ppb. At termination of the experiment (after 4 repeated applications of pesticide), the concentration of chlorpyrifos in the sediment was 443 ppb. Because many frog tadpoles feed in the substrate and are detritivores, this pathway may be a more important contributor than pesticide residues in the water column.

Table 8. Concentration of Carbaryl detected in tanks over time.

Time	Concentration (ppb)	
	sediment	no sediment
0 hours	832	832
24 hours	12.44	15.43
72 hours	nd	12.65
1 week	nd	nd

Histopathology

An histological protocol was developed involving fixation in Bouin's Fluid and paraffin embedding that allows for the serial sectioning of whole amphibian larva. From the *Pseudacris* trial one specimen per tank (N=48) was embedded. Likewise, two specimens per tank (N=96) were embedded from the *Bufo* trial. Specimens were coded by number in a single blind experimental design. One additional control tank was maintained for histological purposes.

Summary: Egg hatching trials using the insecticides were completed and results indicate differences in species sensitivity to these pesticides. High concentrations of carbaryl negatively affect hatching success of *Bufo* and *Ambystoma*, and high concentrations of all pesticides increase deformities among *Pseudacris* hatchlings. Data from larval trials on *Pseudacris* and *Bufo* indicate that pesticide concentration has significant effects on survival, growth, and time to metamorphosis of tadpoles. Sediment analysis indicates that pesticide added to the water column becomes concentrated in the sediment.

Field Studies

The goals of our field study are to design artificial wetlands that will facilitate colonization by a diverse assemblage of amphibians and to develop a protocol to establish and monitor new breeding populations of rarer amphibians. To quantify the success of our project we defined the following objectives:

- Evaluate the success of translocated egg masses into mitigated and existing wetlands
- Evaluate site fidelity of translocated individuals
- Establish breeding populations of target species at Rocky Gap State Park (RGSP)
- Evaluate the suitability of designed wetlands for amphibian colonization by local populations
- Compare the colonization of experimental ponds to ponds at the Rocky Gap Golf Course (RGGC).

In the spring of 1998, a total of 798 *A. jeffersonianum* eggs (114-145 eggs/pond) and 670 *Pseudacris triseriata feriarum* eggs (108-115 eggs/pond) were translocated to experimental ponds at RGSP. By keeping eggs in enclosure bags until larvae had hatched and became strong swimmers, we were able to estimate percent hatching success.

Additional egg masses of *A. jeffersonianum* were kept in enclosure bags at the breeding site from which translocated masses were taken. These additional masses were also monitored for hatching success to determine if percent hatching at RGSP was comparable to percent hatching at an established breeding site. Although estimates of percent hatching success were lower at experimental ponds than from an established breeding site, even the lowest estimates of percent hatching were above 90%. Estimates of percent hatching for *P. triseriata* ranged from 96% - 64%.

Although ponds are enclosed by drift fencing with pitfall and funnel traps, no translocated individuals from the fall of 1998 have been recaptured. This is to be expected for *A. jeffersonianum* because they are fossorial and are not normally seen outside of breeding migrations. Because juveniles can take several years to reach adulthood, we would not expect to find marked individuals returning to ponds at RGSP until spring of 1999 at the earliest.

Metamorphosed larvae from spring translocation efforts have been captured by pitfall traps, funnel traps, and time constrained searches. Captured individuals were measured and marked via toe clipping and/or freeze branding.

Experimental ponds, as well as ponds located on the RGGC, have been monitored for natural colonization by local amphibian species. Egg masses found have been identified to species, total number of eggs has been estimated, and location of deposition within experimental ponds has been mapped. Larvae that have been dipnetted have also been identified to species. In the spring and summer of 1998 the following species had colonized experimental ponds: *Rana clamitans* (green frog), *Rana sylvatica* (wood frog), *Bufo americanus* (American toad), *Pseudacris crucifer* (spring peeper), *Hyla versicolor* (gray treefrog), and *Notophthalmus viridescens* (red-spotted newt). Egg masses and/or larvae of the following species have been found on ponds associated with the golf course: *Rana catesbeiana* (bullfrog), *R. clamitans*, *B. americanus,* and *P. crucifer*. Although the species composition of experimental and golf course ponds seem similar, several important distinctions should be clarified. The absence of *R. catesbeiana* colonization in experimental ponds should aid in the colonization of smaller species of frogs because *R. catesbeiana* has been implicated in the local extirpation of smaller species due to predation. In addition, ponds associated with the golf course seem not to be colonized as ubiquitously by smaller frog species (Table 9) as experimental ponds are. For instance, not only have we failed to find evidence of *H. versicolor* or *R. sylvatica* breeding in golf course ponds, but

larvae of *P. crucifer* have only been found in only one golf course pond while all experimental ponds have contained them.

Table 9. Presence of species colonization: Numbers represent the fraction of ponds that have shown evidence of colonization by each species.

Species	Experimental Ponds	Golf Course Ponds
Rana catesbeiana	0/6	2/3
Rana clamitans	5/6	2/3
Rana sylvatica	1/6	0/3
Pseudacris crucifer	6/6	1*/3
Hyla versicolor	3/6	0/3
Bufo americanus	2/6	1*/3
Notophthalmus viridescens	1/6	0/3

* Only golf course pond containing a shallow shelf of water.

The design of our experimental ponds may promote colonization success of some species. Nearly 60% of egg masses have been deposited on narrow pond shelves which were designed to support vegetation that, in part, provides structure for the ovoposition of amphibian egg masses. Similarly, the one golf course pond constructed with a shallow shelf on its perimeter is the only course pond that has shown evidence of *B. americanus* and *P. crucifer* colonization.

Summary: Egg masses of two species of amphibians have been translocated into experimental ponds at Rocky Gap State Park. Hatching success has been monitored in the egg masses and metamorphosed individuals of both species have been captured and marked for future identification. Experimental ponds, as well as golf course ponds, have been monitored for natural colonization of amphibian species. We have detected six amphibian species that use experimental ponds for breeding and we have detected four species that use golf course ponds for breeding. More importantly, the species composition of our ponds suggests that golf course ponds lack the colonization of smaller species of frogs, while they support the colonization of a large species (i.e. *R. catesbeiana*) that preys on (and could extirpate) smaller species.

Conferences

In order to share the results of our work with the scientific community and to receive additional feedback to improve our protocols, several scientific conferences were attended. From April 16-18, a paper on our field study protocol was presented at the conference for the American Society of Southeastern Biologists at Northeastern Louisiana University. From July 16-22, we attended the 78th annual meeting for the American Society of Ichthyologists and Herpetologists at the University of Guelph in Ontario, Canada. A poster was presented on the protocol and results of our laboratory study as well as a paper on the protocol and preliminary results of our field study.

Budget Expenditures

	Spent or Encumbered
Salary	
P.I. (two months summer salary)	$10,600
Graduate Students (Stipend) $2,500 X 4	$10,000*
Field Assistants $6.25/hr	$667
Fringe Benefits (8% of salaries)	$1701
Travel	$2436
Equipment and Supplies	$3767*
Subcontract for chemical testing (TIWET)	$4290*
Indirect Costs	$3087
Total Spent or Encumbered	$36,548
1997 Money not spent at the time of the last annual report	-1,367
Total from 1998 renewal	$35,181
Remaining to be spent or encumbered prior to renewal	$199

*Because of suspected shortfall we supported three graduate student stipends during the fall semester but will only support one during the spring. Melissa Doris, who has worked with Dr. Morton, is leaving our program and James Julian has agreed to teach next semester and assist me. The Biology department has agreed to support him spring semester. Both Shannon and James Julian will be on research stipends in the summer and Dr. Morton is seeking a replacement for Melissa Doris to continue that aspect of the work. Part of the reason for the shortfall was a clerical error in original calculation of the 1998 budget and, in addition, we had unexpected costs in supplies and in our subcontracts for chemical testing.

Future work

Laboratory Studies-We have completed experiments on four egg hatching trials and two larval growth trials. We are currently completing the last larval growth experiment testing the effect of the three insecticides; Carbaryl, Chlorpyrifos and Imidaploprid. In January we will begin the first of our experiments on three fungicides. Those trials will mirror those already been completed on insecticides. We anticipate completing all the egg hatching trials and at least two larval growth trials on the fungicides by our November report in 1999. In addition, Dr. David Morton is directing a histopathological study on the insecticides based on animals preserved during our previous trials.

Field Studies-The field season in 1999 should be our most informative. We have introduced three species of amphibians in our constructed wetlands that are not found in the immediate area and they should be mature enough to breed in spring 1999. The recovery of marked animals that were intentionally released in 1997 and 1998 and in breeding condition will validate our attempt to establish breeding populations of less common amphibians in these created wetlands. In addition, our monitoring of the adjacent wetlands on the golf course and natural colonization will provide valuable information on the effect of habitat succession on relative species diversity in these sites.